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The Diversity of Legume-Nodulating Bacteria from Several Agroecosystems in Sumberjaya, Lampung

Sri Wedhastri[1*], Yuliana Prahastiwi[1], Jaka Widada[1], Donny Widianto[1], and Siti Kabirun[1]

[1] Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Bacteria that capable of forming root nodules on legumes are known as Rhizobia. They have also known as Legume- Nodulating Bacteria (LNB). They can fix nitrogen from the atmosphere. Diversity of Legume-Nodulating Bacteria is affected by biotic factors (such as their genetic factors, plants, and competition with the other soil microbes) and abiotic factors (such as land use, soil's temperature, pH, chemistry and soil's properties). The aim of this experiment is to know the diversity of eleven Legume- Nodulating Bacteria based on their phenotypic and genotypic characters. The eleven LNB used in this experiments were isolated from several agroecosystems in Sumberjaya, Lampung. The analysis of these LNB diversity were carried out by characterizing both phenotypic and genotypic properties. The diversity analysis showed that the eleven LNB isolates had high diversity, based on nodule formation, and classified into two groups of cross inoculation group.

Key words: Rhizobia, phenotypic diversity, genotypic diversity

Introduction

Bacteria that capable of forming root nodules on legumes are known as Rhizobia (Barcellos *et al*., 2007). They have also have known as Legume-Nodulating Bacteria (LNB) (Mahdi *et al*., 2011). There are currently 98 species in 13 genera of Rhizobia which include genera *Rhizobium, Mesorhizobium, Ensifer, Burkholderia, Bradyrhizobium, Phyllobacterium, Microvirga*, *Azorhizobium*, *Ochrobactrum*, *Cupriavidus, Methyllobacterium, Devosia* and *Shinella* (Weir, 2012).

Diversity of Rhizobia can be analyzed by characterizing their phenotypic and genotypic properties (Naz *et al*., 2009). Phenotypic characterization of Rhizobia can be carried out by identifying their cell's morphology, colony's morphology, cell's biochemical properties, their ability to form root nodules (cross inoculation group) and nitrogen fixation (Boncher, 2009; De Bruijn 1992). Although phenotypic characterization is helpful in the identification of soil rhizobia diversity, the identification based on the molecular approaches believed to be more accurate (Naz *et al*., 2009). One of the molecular techniques that can be used to quickly identify the microbial diversity is by using Repetitive Extragenic Palindromic Polymerase Chain Reaction (REP-PCR) (Beyer *et al*., 1998). Diversity of Legume-Nodulating Bacteria in the soil is affected by biotic factors such as cross inoculation group and abiotic factors such as land management (Madigan *et al*., 2003; Purwaningsih, 2009).

***Corresponding author:**
Sri Wedhastri
Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia, Jl. Flora, Bulaksumur, Sleman, Yogyakarta, 55281, email : wedhastri@faperta.ugm.ac.id

Materials and Methods

Bacterial isolates

USDA122, Tha7, and eleven LNB were isolated from several agroecosystems in Sumberjaya, Lampung which include

UGM4, UGM5, UGM15, UGM19, UGM20, UGM22, UGM26, UGM27, UGM28, UGM35 and UGM39. These isolates are collections of Agricultural Microbiology Laboratory, Faculty of Agriculture, Universitas Gadjah Mada in Yogyakarta.

Phenotypic Characterization of Legume-Nodulating Bacteria (LNB)

Cells and colony's morphology

Characterization of cell morphology include Gram properties, shape and size of the cells, and cell's motility. Bacterial culture in Yeast Mannitol Broth (YMB) (24-48 h) were used for testing Gram staining, shape and size of cells. Cell's motility was examined by using cultures that were grown on semi solid Yeast Mannitol Agar (YMA). Colony's morphology was examined by using cultures that were grown for 24-48 h on YMA.

Cell's biochemical properties

Biochemical properties were examined by testing isolates growth in several media, namely: Congo Red Yeast Mannitol Agar (CRYMA), Bromothymol Blue Yeast Mannitol Agar (BBYMA), and Glucose Pepton Agar (GPA). Bacterial isolates were also tested in the formation of 3-ketolactose, catalase activity, and the use of multiple sources of C and N.

Root nodulation (cross inoculation group) and nitrogen fixation test

To test the ability in root nodulation, the eleven legume-nodulating bacteria isolates were used to inoculate several plants host. The plant hosts used in this work were: siratro (*Macroptilium artropurpureum* DC.), soybean (*Glycine max* L.), red bean (*Vigna angularis*), lamtoro (*Leucaena leucocephala*) and sengon (Albizzia falcataria) using minirhizotron method (made from disposable petridish) (Figure 1).

The ability of nitrogen fixation was examined by using Acetylene Reduction Assay (ARA) (Halbleib and Ludden, 2000).

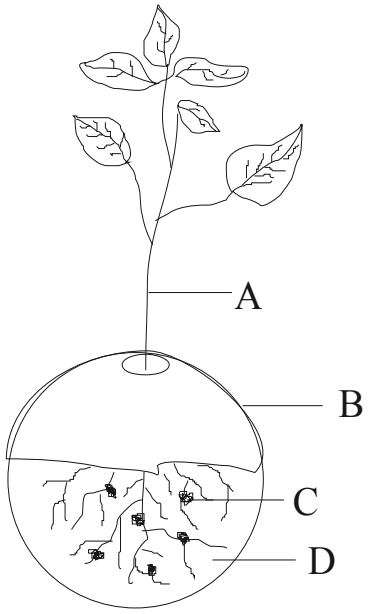

Figure 1. Plant growth scheme with minirhizotron methods A) plants B) Dispossable petridish covered with aluminum foil, C) root nodules, and D) zeolite

Genotypic characterization of Legume-Nodulating Bacteria (LNB).

DNA isolation.

1,5 ml bacterial culture in Nutrient Broth (NB) medium (18-24 h) were transferred into microtube, then centrifuged at 12,000 rpm for 5 min. Supernatant was then discarded and the pellets were dissolved with 410 µl TE (10mM Tris-HCl; 1mM EDTA (pH 8) supplemented with 50 µl lysosim (60 mg/ml) and incubated at 37 °C for 30 min. The solution was added with 30 µl SDS 10% (10 gram in 100 ml sterile distilled water) and 10 µl proteinase-K (20 mg/ml) and incubated at 37 °C for 30 min. It was coupled with 100 µl NaCl 5M and 200 µl solution CTAB 10% in NaCl, then incubated at 65 °C for 10-15 min. After incubation, 600 µl chloroform was added and homogenized, then centrifuged at 12.000 rpm for 5 min. It resulted in a solution with 3 layers. Then the top layer was transferred into new microtubes and isopropanol was added to 500 µl. The mixture was then incubated for 24 h in the refrigerator and centrifuged again at 12,000 rpm for 5 min. Supernatant was discarded and the pellet

was washed with 100 ml 70% ethanol and centrifuged at 12,000 rpm for 5 min and then air dried for over night and redissolved with 50 ml TE buffer solution. The purity of genomic DNA were examined by electrophoresis on 1% Agarose.

REP-PCR.

The reaction mixture contained 1µl DNA genome, 12.5 µl KAPPA PCR kit, 9 µl *nuclease free water* and 2.5 µl primer BOX AIR (5'-CTAC GGCAAGGCAAGGCGACGCTGACGCTGA CG-3'), so the total reaction volume was 25 ml. The reaction mix was placed on a termocycler and subjected to PCR cycles: 95 °C for 4 min., followed by 30 cycles of 94 °C for 30 sec, 51 °C for 1 min. and 65 °C for 8 min, and followed by the final elongation at 65 °C for 8 min. PCR amplified fragments were electrophoresed in an agarose gel (1.5%) for 50 min. and were visualized using ethidium bromide staining.

Result and Discussion

Phenotypic characterization of Legume Nodulating Bacteria (LNB)

Phenotypic characterization of rhizobia was performed by identifying the colony morphology, cell morphology, Gram properties, biochemical properties of cells (Boncher, 2009) and examination of cross-inoculation group bacteria (De Bruijn, 1992). Rhizobium is a Gram-negative, rod-shaped, aerobic, motile and does not form spores. Colonies usually white or cream with circulair shape, convex, semi-transculent or dark and low convex with diameter 2-4 mm at the age of 3-5 days in medium Yeast Mannitol Agar (YMA). The genus Rhizobium bacteria grow at optimum temperature of 25-30 °C at pH 6-7. Almost all bacteria of the genus Rhizobium are able to form root nodules on legume whether or not they perform nitrogen fixation (Kuykendall *et al*., 1889).

Table 1. The observation of colony and cell morphology

No	Colony's morphology						Cell's morphology			
	Isolate	Shape of colony	Ele-vation	The edge of the colony	The structure of the colony	Colony growth on CRYMA	Shape of the cell	Gram	Size of the cell (µm)	Motility
1	USDA122	Circulair	Low convex	Undulate	Coarsely Grannular	red	Rod	negative	0,5 x 0,7-3,1	Motile
2	THA7	Irregulair	Low convex	Undulate	Finely Grannular	white	Rod	negative	0,5 x 1,05-4,2	Motile
3	UGM4	Amoeboid	Low convex	Lobate	Finely Grannular	red	Rod	negative	0,35-0,5 x 1,05-2,1	No Motile
4	UGM5	Circulair	Low convex	Undulate	Finely Grannular	white	Rod	negative	0,5 x 0,7-3,1	No Motile
5	UGM15	Circulair	Low convex	Entire	Finely Grannular	white-yellowish	Rod	negative	0,5 x 0,7-3,1	No Motile
6	UGM19	Amoeboid	Effuse	Ramose	Wavy Entelaced	red	Rod	negative	0,525-0,7 x 1,05-3,1	Motile
7	UGM22	Circulair	Low convex	Entire	Finely Grannular	white	Coccus	negative	0,5 x 0,5	No Motile
8	UGM26	Cirulair	Low convex	Undulate	Coarsely Grannular	pink	Rod	negative	1,05 x 2,1-3,1	No Motile
9	UGM27	Circulair	Effuse	Undulate	Finely Grannular	white	Rod	negative	0,5-1,05 x 1,05-2,1	No Motile
10	UGM28	Circulair	Low convex	Entire	Finely Grannular	white	Coccus	negative	0,5 x 0,5	No Motile
11	UGM35	Circulair	Low convex	Undulate	Coarsely Granular	white-reddish	Rod	negative	0,5-0,7 x 0,7-3,1	No Motile
12	UGM39	Circulair	Effuse	Entire	Coarsely Granular	pink	Rod	negative	0.5-1,05 x 2,1-3,1	Motile
13	UGM20	Circulair	Effuse	Entire	Finely Grannular	red	Rod	negative	0,5 x 2,1-5,25	Motile

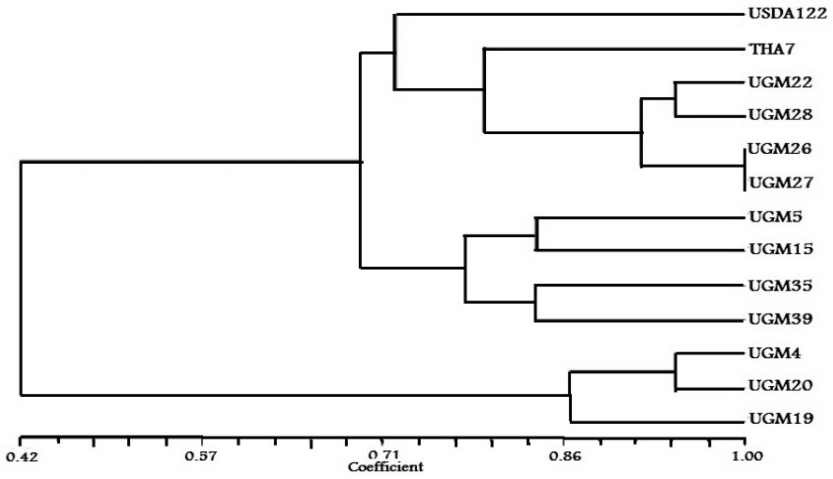

Figure 2. Dendogram of similarity relationship between tested isolates by biochemical tests

Table 2. Ability to form root nodules (cross inoculation group) test

No	Isolates	The number of root nodules				
		Siratro	Soybean	Red Beans	Lamtoro	Sengon
1	USDA122	+	+	-	-	+
2	THA7	+	+	+	-	+
3	UGM4	+	+	-	-	+
4	UGM5	+	+	+	-	+
5	UGM15	+	+	-	-	+
6	UGM19	+	+	-	-	+
7	UGM22	+	+	-	-	+
8	UGM26	+	+	-	-	+
9	UGM27	+	+	-	-	+
10	UGM28	+	+	-	-	+
11	UGM35	+	+	-	-	+
12	UGM39	+	+	-	-	+
13	UGM20	+	+	-	-	+
14	Kontrol (-)	-	-	-	-	-

Both morphological observations of the cells or colonies, indicating that overall the tested bacteria are Gram-negative bacteria to form colonies almost entirely circular and forms the majority of the cells are rod (Table 1).

Biochemical data indicated that all of bacterial isolates tested were from different groups (Figure 2). This was indicated by the low coefficient of similarity. Only isolates UGM26 and UGM27 that indicated a close relationship with similarity coefficient was 1. This indicates that the two isolates were from the same group.

Biochemical data indicated that there was a high diversity of bacterial isolates tested. Judging from the origin of the isolates tested, these isolates came from different agroecosystems. This proved that environmental factors, especially soil tillage systems and different types of crop diversity affect rhizobia in the soil. The evaluation on the ability of root nodule formation showed that there was low cross-inoculation group.

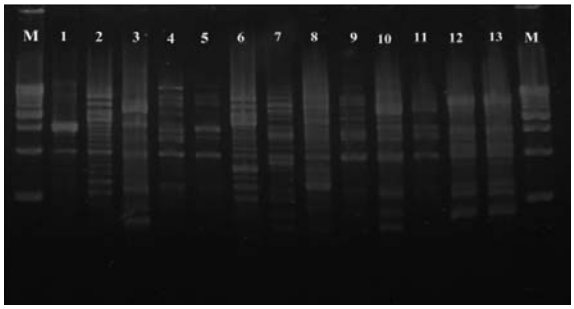

Figure 3. Amplificatin product of REP-PCR in agarose 1,5 % (b/v). M = marker DNA; 1 = UGM4; 2 = UGM5; 3 = UGM15; 4 = UGM19; 5 = UGM22; 6 = UGM26; 7 = UGM27; 8 = UGM28; 9 = UGM35; 10 = UGM39; 11 = UGM20; 12 = USDA122; 13 = THA7

This was indicated by the uniformity of the results shown in the five plants used in testing (Table 2).

Genotypic characterization of Legume-Nodulating Bacteria (LNB).

REP-PCR can be used in the identification and classification of bacterial strains (De Bruijn 1992; Xue-Xian *et al*.,1999). Techniques Repetitive Extragenic Palindromic Polymerase Chain Reaction (REP-PCR) can be used to distinguish microbial diversity based on the number and spacing of its repetitive sequences. REP-PCR data indicated that eleven bacteria tested had a high diversity (Figure 3 and 4).

As the conclusion, the 11 isolates of Legume- Nodulating Bacteria tested had high phenotypic and genotypic diversity. There were two cross-inoculation group, namely: (1) UGM5 were able to form nodules on the plant siratro, soybeans, red beans and sengon, and (2) UGM4, UGM15, UGM19, GMU 22, UGM26, UGM27, UGM28, UGM35, UGM39 and UGM20 were able to form nodules on the plant siratro, soy and sengon.

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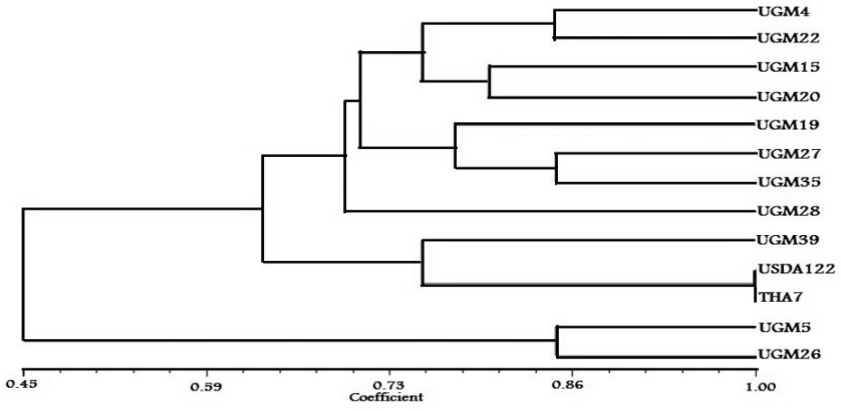

Figure 4. Dendogram of similarity relationship between test isolates by REP-PCR amplification results

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